RAPD Marker Variation among Smooth Bromegrass Cultivars

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ABSTRACT

The level of genetic diversity within and among smooth bromegrass (*Bromus inermis* Leyss.) cultivars and land races is unknown. The objective of this study was to investigate and characterize genetic diversity of smooth bromegrass cultivars and selected populations on the basis of random amplified polymorphic DNA (RAPD) markers. Variation among 277 individual plants from 40 smooth bromegrass cultivars was evaluated by means of RAPD markers. Nineteen primers evaluated amplified 97 informative amplicons. Several RAPD marker bands showed unique patterns of mean frequency differences among germplasm groups. A dendrogram constructed from average linkage cluster analysis did not indicate any clear pattern of division on the basis of discrete or putative climatype or adaptation zones. There was considerable correspondence to known pedigree relationships revealed from a previous smooth bromegrass morphological clustering analysis, particularly for lines that are closely related to each other. The two interspecific hybrids between *B. inermis* and *B. pumpellianus* Schribner (Polar) and *B. riparius* Rehm. (S-9183-H) did not exhibit any species-specific markers. All groups of smooth bromegrass germplasm were found to have high within-population genetic variation that ranged from 84 to 96% of the total, reflecting the outcrossing reproduction and probably the complex inheritance of smooth bromegrass. Analysis of molecular variance showed the largest interpopulation genetic variation for contemporary germplasm sources, supporting morphological studies for the existence of genetic variability among contemporary smooth bromegrass germplasms. These results suggest that landrace cultivars likely remain useful for germplasm improvement and cultivar development.

Smooth bromegrass cultivars grown in North America originated from eastern European and temperate Asian germplasm sources collected in the second half of the 19th century. Before the 1950s, most cultivars were released as direct increases of introduced ecotypes or as naturalized selections of these ecotypes (Casler and Carlson, 1995). In vitro dry matter digestibility (IVDMD) and disease resistance of smooth bromegrass were improved markedly during the latter half of the 20th century, but little is known about genetic diversity among land races and contemporary germplasms of smooth bromegrass. Although numerous smooth bromegrass cultivars have been developed by intensive selection and breeding efforts since the 1950s, phenotypic data suggests that there has been little change in forage yield (Casler et al., 2000, 1996; Casler and Vogel, 1999).

The commonly grown form of smooth bromegrass is a polysomic octoploid, $2n = 8x = 56$, with a genome structure of $AAAAB_1B_1B_2B_2$ (Ghosh and Knowles, 1964; Armstrong, 1980, 1992). One explanation for the slow progress in genetic improvement in breeding smooth bromegrass is its complex polyploid nature. Because of this, the gradual release of new recombinants and transgressive segregants over many generations is very slow, limiting the release of new genetic variability and the potential for genetic gains (Casler et al., 2000).

Breeding can be optimized and accelerated by thoroughly screening, evaluating, and classifying germplasm. Smooth bromegrass germplasm is classified by three climatypes: meadow, steppe and intermediate (Casler and Carlson, 1995; Vogel et al., 1996). The meadow or northern type is characterized by slow spreading, open sod, prostrate growth, and a rachis:canopy height ratio of 1.5. The steppe or southern type is characterized by superior seedling vigor and ease of establishment, deep roots, drought and frost tolerance, short and narrow leaves, erect growth, rachis:canopy height ratio of 2, and compact panicles. The intermediate type is a hybrid between the two main climatypes meadow and steppe.

Degree and pattern of temperature and rainfall vary in geographically defined zones. Smooth bromegrass cultivars have shown inconsistent forage yield ranking across a wide range of geographic locations (Casler et al., 2000). Similar pedigrees, selection history, and selection location could explain much of the geographic grouping and adaptation characteristics of cultivars. Casler et al. (2001) showed that cultivars could be clustered into groups that reflect differential mean performance as well as differential adaptation among zones within the target region. Smooth bromegrass cultivars and populations are grouped in latitudinal and longitudinal adaptation zones, based on where cultivars and populations were developed, collected, or increased in North America.

Little is known about the genetic relatedness of smooth bromegrass cultivars in these different groupings, particularly the level of genetic diversity among land races and contemporary cultivars. Morphological variation may not reliably reflect the real genetic variation because of genotype-environment interaction and largely unknown genetic control of polygenically inherited morphological and agronomic traits (Smith and Smith, 1992). Thus, DNA variation may provide another useful measure of genetic changes in smooth bromegrass.

An important research application of molecular marker technology has been in the measurement of genetic diversity and genetic relationships among individuals and populations. Molecular markers have assisted the analysis of plant genomes, providing useful data for many studies for a wide variety of plant species. Changes in marker frequencies associated with changes in population performance have been reported (Stuber

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and Moll, 1972; Stuber et al., 1980). Ferdinandez et al. (2001) used RAPD markers to demonstrate that a hybrid population involving smooth bromegrass was genetically intermediate to its parents, smooth bromegrass and meadow bromegrass (*B. riparius*), but closer to smooth bromegrass, reflecting selection toward the smooth bromegrass phenotype.

This study consisted of 40 smooth bromegrass cultivars and experimental populations. The objective was to investigate and characterize relatedness among smooth bromegrass cultivars and breeding populations, on the basis of RAPD markers, identifying sources of variation associated with RAPD markers.

MATERIALS AND METHODS

Germplasm

Forty smooth bromegrass cultivars and experimental populations developed, collected, or increased in North America were included in the experiment (Table 1). Selection of specific cultivars, experimental populations, and the total number of entries with their distribution in North America were based on availability and germination of seed. All cultivars were represented by certified seed, and all experimental populations were represented by Syn-2 or later seed from breeders' increases.

Smooth bromegrass cultivars and populations were sown, at 10 plants per population, in spring 1998. Greenhouse management involved watering, pest control, occasional clipping, and nitrogen fertilization to stimulate seedling growth.

DNA Isolation

The DNA was extracted from three to 10 individual seedlings from each population as described in Skroch and Nienhuis (1995). Approximately 0.5 to 0.75 g of fresh tissue was harvested and ground with 500 mL of potassium ethyl xanthogenate (PEX) (Sigma-Aldrich, St. Louis, MO) at maximum speed of 5.0 m s^{-1} for 40 s with the Bio 101 (Vista, CA) Savant FP120 Fast PrepTM. After grinding, tissue was transferred to centrifuge tubes and allowed to incubate for 30 min in a 65°C water bath. After organic and aqueous phases of the extraction mixture were separated by centrifugation (Eppendorf 5415C microfuge), nucleic acids were precipitated by adding a 6:1 mixture of 95% (v/v) ethanol and 7.5 *M* ammonium acetate. After removing RNA (by means of 100 mg/mL RNase A for

Table 1. Origins and phenotypes of smooth bromegrass cultivars or populations.

Cultivar or population	N†	CT‡	Origin§	Latitude¶	Longitude¶	IVDMD#
WB88S-Tu	10	M	Russia			No
WB88S-Ch	6	M	Russia			No
WB88S-Ka	5	M	Russia			No
Lincoln††	8	S	Nebraska	South	West	No
Lancaster††	8	S	Nebraska	South	West	No
Manchar††	10	I	Washington	North	West	No
Carlton	5	M	Saskatchewan	North	West	No
Magna	8	I	Saskatchewan	North	West	No
Lyon††	6	S	Nebraska	South	West	No
Beacon	8	S	Iowa	South	East	No
Saratoga††	4	S	New York	North	East	No
Sac	6	S	Wisconsin	North	East	No
York	4	S	New York	North	East	No
Radison	5	S	Saskatchewan	North	West	No
Signal	5	I	Saskatchewan	North	West	No
Alpha	4	S	Wisconsin	North	East	Yes
Badger	7	S	Wisconsin	North	East	Yes
Lincoln-HDMD-C3	10	S	Nebraska	South	West	Yes
Lincoln-HDMDYD-C3	7	S	Nebraska	South	West	Yes
NE-B1-1	8	S	Nebraska	South	West	Yes
NE-B1-2	8	S	Nebraska	South	West	Yes
PL-BDR1	8	S	Pennsylvania	South	East	Yes
WB19e	8	S	Wisconsin	North	East	Yes
WB20e	7	S	Wisconsin	North	East	Yes
WB10-hDS	9	S	Wisconsin	North	East	Yes
Palmer	6	M	Alaska	North	West	No
Elsberry††	8	S	Iowa	South	East	No
Homesteader††	5	I	South Dakota	North	West	No
Martin††	6	M	Minnesota	North	East	No
Achenbach††	8	S	Hungary	South	West	No
Fischer††	7	S	Iowa	South	East	No
Redpatch	10	S	Ottawa	North	East	No
Fox	5	S	Minnesota	North	East	No
Southland††	7	S	Kansas	South	West	No
Baylor	8	S	Iowa	South	East	No
Bravo	7	M	Ontario	North	East	No
Polar	7	H	Alaska	North	West	No
Mandan 404††	8	M	North Dakota	North	West	No
S-9183-H	3	H	Saskatoon	North	West	No
Jubilee	8	M	Ontario	North	East	No

† N = number of plants.
‡ CT = climatype: *M* = meadow (northern), S = steppe (southern), I = intermediate, H = interspecific hybrid.
§ State, province, or country where smooth bromegrass population was developed, collected, or increased.
¶ North = population developed or increased north of 42°N latitude in North America, South = population developed or increased south of 42°N latitude in North America, East = population developed or increased east of 95°W latitude in North America, West = population developed or increased west of 95°W latitude in North America.
# Population selected or not selected for increased in vitro dry matter digestibility (IVDMD).
†† Land race cultivars: original cultivars released as seed increases or naturalized ecotypes.

1 h at 37°C) and any remaining debris, DNA was reprecipitated by the addition of 10:1 solution of ethanol and 3 *M* sodium acetate. After a 70% (v/v) ethanol wash and pelleting, DNA was hydrated in TE buffer (1 m*M* Tris, pH = 8.0, 0.1 m*M* EDTA, pH = 8.0). DNA concentrations were quantified in a logical numerical order with a Hoefer Scientific TKO-100 Fluorometer (Amersham Pharmacia Biotech, Piscataway, NJ).

RAPD Reactions

RAPD reactions were performed as described in Johns et al. (1997) in an M.J. Research, Inc. (Waltham, MA) PTC-100 Programmable Thermal Controller. Cycling temperature settings were 91°C for denaturation, 42°C for annealing, and 72°C for elongation. In the first cycle, cycling times were 60 s for denaturation, 15 s for annealing, and 70 s for elongation. During the subsequent 39 cycles, denaturation was set for 15 s, annealing for 15 s and elongation for 70 s.

Polymerase chain reaction (PCR) amplifications were performed in a final reaction volume of 10 mL, containing the following reaction buffer: 50 m*M* Tris, pH 8.5, 20 m*M* KCl, 2 m*M* $MgCl_2$, 500 mg/mL of bovine serum albumin (BSA), 2.5% (v/v) ficoll 400, and 0.02% (w/v) xylene cyanol. Reactant concentrations were 100 m*M* dNTPs (deoxy nucleotide triphosphates) (Promega, Madison, WI), 2 ng/mL of DNA template, 0.4 m*M* of decamer primer Operon Technologies, Inc. (Alameda, CA); University of British Columbia, (Vancouver, BC, Canada), and 0.6 unit (5 units/mL) of *Taq* DNA Polymerase (Promega, Madison, WI). All RAPD reaction products were electrophoresed in 20 cm × 25 cm, 1.5% (w/v) agarose gels in 1× TBE (Tris, Boric Acid, EDTA) buffer. Gels were run for 2 h at 300 V in Gibco/BRL Life Technologies (Invitrogen, Carlsbad, CA) H4 gel apparatus, stained with ethidium bromide, and illuminated by UV light and subsequently photographed with Polaroid 667 film.

Primer Screening

One hundred decamer oligonucleotide primers from Operon Technologies, Inc. (primer kits A, AE, AF, and AG) and University of British Columbia (UBC series) were initially screened for polymorphisms against a subset of 48 smooth bromegrass plants representing 20 of the 40 populations in this study. Seventeen Operon Technologies, Inc. primers and two UBC primers, highly polymorphic among cultivars, were used to amplify all 277 individuals plants from each cultivar and population.

Data Collection and Statistical Analysis

The frequency of 153 polymorphic RAPD markers (scored as 1 for presence of the amplicon or 0 for absence of the amplicon for each individual plant and stored as binary matrix) was computed for the 40 populations. A homogeneity χ^2 test was performed on frequencies of 153 RAPD markers to assess the amplicons that discriminated most among the 40 populations. Ninety-seven amplicons highly significant ($P < 0.01$) for the homogeneity test were selected for further analysis in the study.

The GENMOD and LOGISTIC methods for analysis of binomial data (SAS, 1999) were applied to the RAPD data for each of four different germplasm groupings of smooth bromegrass, to determine if phenotypic diversity based on RAPD markers showed any associations with climatypes, adaptation zones in North America, or selection for digestibility (Table 1). Molecular markers should be randomly distributed across geographic zones, climatypes, and selection lines, unless they have a selective advantage per se or are linked to loci with a selective advantage. Use of a severe *P*-value (0.01) should allow for confident identification of markers for these linkage blocks related to geographic zones, climatypes, and selection for digestibility. Linkage mapping will be required to confirm the presence of these putative linkages, but the presence of associations should lead to identification of potentially useful markers. The GENMOD and LOGISTIC analyses were also used to compute least-squares means of marker frequencies in each germplasm group. Contrasts for analysis of binomial data (SAS, 1999) were applied to test for differences among groups within the four germplasm groupings (Table 1).

From the 97 polymorphic RAPD markers transformed into a binary matrix, a pairwise Jaccard similarity coefficient matrix was computed by means of NTSYS-PC 2.01 (Rohlf, 1997) on all individuals across populations. The similarity matrix $\boldsymbol{S}_{ij}$ (similarity between individual plants i and j) was converted to a Euclidean distance matrix by the elementwise formula $(1 - S_{ij})^{0.5}$. Euclidean distances, converted from Jaccard similarity coefficients, were used as the measure of genetic distance between all individuals.

Analysis of molecular variance (AMOVA; Excoffier et al., 1992; Schneider et al., 1997) was performed on all 277 individuals, partitioning the Euclidean distance matrix into three sources of variation: among groups, among populations within groups, and within populations. Variance components were estimated by equating AMOVA mean squares to their expectations. Variance components were tested by nonparametric permutation tests (Schneider et al., 1997).

Cluster analysis, based on the unweighted pair-group method of arithmetic averages (UPGMA; SAS, 1999), was used to construct a distance dendrogram for the 40 populations. Concordance between the morphological-agronomic distance matrix of Casler et al. (2000) and the molecular distance matrix was measured by matrix correlation (Mantel, 1967). A confidence interval for the Mantel correlation was generated from 999 random permutations of the RAPD marker distance matrix (Smouse et al., 1986).

RESULTS AND DISCUSSION

RAPD Polymorphisms

A total of 100 primers were initially screened, revealing a large number of amplicons varying in size and intensity. Only a subset of 19 primers revealing polymorphic markers was used for the population assays. A total of 153 polymorphic markers were scored from the 19 primers. Ninety-seven markers that demonstrated frequency differences among populations were selected by the homogeneity χ^2 test ($P < 0.01$) on marker frequencies of the 153 RAPD markers. The number of markers scored per primer ranged from 4 to 14. The scored markers were comprised of fragment lengths ranging from 300 to 1600 base pairs, and differences in amplicon intensities were not considered in the data analysis.

Marker Variation among Germplasm Groups

No population-specific or species-specific fragments were detected in the 40 smooth bromegrass cultivars and populations. As with other cross-pollinating species (Phan, 2000), no two individuals were found to be identical by RAPD markers. Ferdinandez et al. (2001) reported a UBC680.2079 band found in all meadow bromegrass (cv. Fleet) individuals, but absent in smooth

Table 2. Mean frequency and P-values for 19 RAPD bands that showed statistically significant variation among smooth bromegrass germplasm groups (climatypes and interspecific hybrids).

					Comparisons			
Primer–band	Steppe	Meadow	Intermediate	Hybrid	Groups†	Meadow vs. steppe‡	Meadow and steppe intermediate‡	Smooth bromgrass vs. Hybrids‡
A18.1300	0.650	0.459	0.456	0.524	0.0068	0.0045	0.1274	0.5296
A18.1100	0.620	0.456	0.400	0.691	0.0082	0.0198	0.0467	0.2173
AE04.2100	0.579	0.534	0.213	0.619	0.0076	0.8255	0.0008	0.3568
AE04.0900	0.659	0.612	0.413	0.310	0.0042	0.2275	0.0114	0.1161
AE06.1200	0.901	0.569	0.681	0.857	<0.0001	<0.0001	0.1497	0.6363
AE10.1400	0.490	0.640	0.344	0.834	0.0065	0.0435	0.0194	0.0334
AE16.1800	0.847	0.666	0.763	0.643	0.0028	0.0015	0.6761	0.1129
AE18.1300	0.684	0.424	0.588	0.619	0.0023	0.0004	0.4415	0.7680
AE18.1200	0.339	0.591	0.656	0.691	<0.0001	0.0002	0.0443	0.2426
AE18.1100	0.840	0.390	0.925	0.929	<0.0001	<0.0001	0.0111	0.2870
AF06.0700	0.384	0.640	0.500	0.667	0.0061	0.0083	0.4328	0.1037
AF10.0750	0.474	0.659	0.794	0.691	0.0009	0.0074	0.0191	0.6682
AF14.1000	0.931	0.736	0.675	0.548	<0.0001	<0.0001	0.0090	0.0393
AF14.0900	0.253	0.585	0.494	0.929	<0.0001	<0.0001	0.4057	0.0231
AF14.0875	0.738	0.473	0.675	0.691	0.0022	0.0002	0.9630	0.5493
AG14.0950	0.886	0.716	0.900	0.072	<0.0001	0.0040	0.1521	0.0002
UBC204.1550	0.833	0.712	0.763	0.143	0.0008	0.0187	0.8377	0.0017
UBC204.1050	0.663	0.383	0.500	0.619	0.0006	0.0002	0.2292	0.5853
UBC318.0600	0.747	0.387	0.638	0.691	<0.0001	<0.0001	0.6418	0.4455

† 3 df.
‡ 1 df.

bromegrass (cv. Signal). In addition, 85% of the interspecific hybrid individuals between meadow bromegrass and smooth bromegrass exhibited this meadow bromegrass specific marker. In their study, phenetic analysis of the populations utilized differences in marker frequencies. Previous RAPD studies used species-specific markers and marker frequency differences to determine species relationships and cultivar identification (Kangfu and Pauls, 1993; Gherardi et al., 1998; Phan, 2000; Ferdinandez et al., 2001).

Nineteen RAPD marker bands showed statistically significant ($P < 0.01$) variation among all smooth bromegrass germplasm groups (climatypes and hybrids) (Table 2). Of these, 14 markers showed frequency differences ($P < 0.01$) in the contrast of meadow vs. steppe. In the contrast of meadow and steppe climatypes versus intermediate, only two of these markers demonstrated frequency differences ($P < 0.01$). For the contrast of smooth bromegrass populations versus the two interspecific hybrids, only two of these markers demonstrated frequency differences ($P < 0.01$). Those three different contrasts revealed many fragments having a unique pattern of frequency differences in specific smooth bromegrass germplasm groups. Markers having unique frequency patterns among smooth bromegrass groups may be useful in classifying germplasm, identifying potential heterotic materials, and grouping germplasm of similar genetic background.

Thirteen RAPD markers had significant ($P < 0.01$) variation between northern vs. southern origins of smooth bromegrass (Table 3). Among these markers, 10 had a pattern of higher mean frequencies for smooth bromegrass populations developed north of 42°N latitude in North America. Only three markers showed mean frequency differences ($P < 0.01$) between east and west origins of smooth bromegrass germplasm (Table 4), indicating that latitude-related factors are more important than longitude-related factors in discriminating among smooth bromegrass germplasms. Sixteen RAPD markers revealed significant ($P < 0.01$) frequency differences between smooth bromegrass populations selected vs. unselected for IVDMD (Table 5). Most of the marker bands showing statistically significant variation between germplasm groups had high mean frequency for populations of smooth bromegrass selected for IVDMD. Markers AE08.1100, AF10.0750, and UBC204.0780 had a particular pattern of low frequency in smooth bromegrass populations selected for IVDMD.

Marker bands depicting unique patterns in their mean frequency toward specific smooth bromegrass germ-

Table 3. Mean frequency and *P*-value for 13 RAPD bands that showed statistically significant variation between smooth bromegrass populations developed in northern vs. southern adaptation zones of North America.

Primer–band	North†	South†	*P*-Value
AE04.2100	0.450	0.663	0.0041
AE04.0800	0.984	0.841	0.0015
AE10.0800	0.910	0.697	<0.0001
AE16.0800	0.482	0.706	0.0007
AE18.1300	0.570	0.735	0.0049
AE18.1200	0.560	0.263	<0.0001
AE18.0800	0.596	0.453	0.0012
AF06.1050	0.475	0.353	0.0071
AF06.0950	0.791	0.563	<0.0001
AF06.0700	0.543	0.335	<0.0001
AF14.0900	0.430	0.250	0.0004
AG10.0800	0.375	0.247	0.0066
AG14.0825	0.878	0.670	<0.0001

† Origin north or south of 42°N latitude in North America.

Table 4. Mean frequency and *P*-value for three RAPD bands that showed statistically significant variation between smooth bromegrass populations developed in eastern vs. western adaptation zones of North America.

Primer–band	East†	West†	*P*-Value
AE12.0900	0.617	0.448	0.0016
AE16.1800	0.895	0.730	0.0007
AG10.0725	0.227	0.095	0.0022

† Origin east or west of 95°W latitude in North America.

Table 5. Mean frequency and *P*-value for 16 RAPD bands that showed statistically significant variation between smooth bromegrass populations selected or not selected for increased in vitro dry matter digestibility (IVDMD).

Primer–band	Selected	Not selected	*P*-Value
A18.1400	0.607	0.789	0.0038
AE04.0950	0.745	0.594	0.0049
AE04.0900	0.747	0.559	0.0009
AE08.1100	0.316	0.829	<0.0001
AE16.1800	0.895	0.752	0.0080
AE18.1300	0.800	0.550	0.0005
AE18.1100	0.892	0.705	0.0012
AF10.0750	0.346	0.630	0.0002
AF14.1000	0.988	0.794	0.0029
AF14.0875	0.813	0.622	0.0021
AG14.0825	0.695	0.841	0.0022
UBC204.1300	0.898	0.689	0.0005
UBC204.1050	0.745	0.527	0.0006
UBC204.0780	0.440	0.638	0.0029
UBC318.2000	0.722	0.451	<0.0001
UBC318.0600	0.882	0.573	<0.0001

plasm groups provided an indication of associations between these markers and alleles related to phenotype or geographic origin. These results suggest that DNA markers could be useful in identifying quantitative trait loci controlling IVDMD and geographic adaptation traits in controlled experimental crosses. The frequency differences between IVDMD groups and geographic origins support phenotypic results from previous studies, demonstrating genetic gains in IVDMD (Casler et al., 2000) and genetic variation for adaptation (Casler et al., 2001).

These results are consistent with previous research based on molecular marker frequencies in other species. Changes in marker frequencies associated with changes in population performance have been reported in maize (*Zea mays* L.) (Stuber and Moll, 1972; Stuber et al., 1980). Strelchenko et al. (1999) used RAPD marker variation to associate genetic differentiation with geographical distribution of barley (*Hordeum* L.) germplasm. Li et al. (2001) found patterns of RAPD marker diversity associated with the major ancestors of USA and Chinese soybean [*Glycine max* (L.) Merr.] cultivars, reflecting the geographical origin of the lines. RAPD marker variation was successfully used to identify cultivated races of sorghum [*Sorghum bicolor* (L.) Moench] and regions with maximum genetic diversity (Menkir et al., 1997). RAPD marker variation patterns have been associated with regions of origin of sorghum germplasm, broadly concordant with previous clustering patterns obtained using morphological characters, in which regions with broad-climatic conditions were grouped together (Ayana et al., 2000). Huff et al. (1993) used RAPD marker variation to characterize buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.] populations according to the geographic origin.

Genetic Diversity of Populations

The RAPD variability was relatively high among the 40 smooth bromegrass cultivars and populations, reflecting the phenotype of a dominant marker in highly heterozygous populations (Table 6). All groups of smooth bromegrass germplasm were found to have high within-population genetic variation and the proportion among populations ranged from 4.0 to 16.1%. The contemporary germplasm sources, consisting mainly of diverse cultivars and experimental populations, had the largest inter-population genetic variation of 16.1%, while the between-population variation for land race cultivars was 11.4% (data not shown). These results suggest slight differences in marker variation between natural ecotypes and experimental germplasm probably reflecting limited cycles of selection in the development of smooth bromegrass contemporary germplasm sources. In general, similar and significant levels of interpopulation RAPD genetic variation were found for the different smooth bromegrass germplasm groups.

The intergroup RAPD variation was low, ranging from 0.1% (land race cultivars vs. contemporary germplasm sources) to 3.6% (smooth bromegrass vs. interspecific hybrids) (Table 6). The intergroup variation for land race cultivars vs. contemporary germplasm sources was not statistically significant, suggesting little overall difference in RAPD markers between smooth bromegrass contemporary germplasm sources and land race cultivars. Slow progress in breeding improved cultivars of smooth bromegrass has been reported by Casler et al. (2000). No cultivar was derived from more than three cycles of selection and recombination from essentially wild or natural germplasm, and most cultivars represent one cycle of selection or no selection history (Alderson and Sharp, 1994; Hanson, 1972; Casler et al., 2000). Lack

Table 6. Analysis of molecular variance (AMOVA) sum of squares partitioning of total RAPD marker variation into pairwise comparisons among groups, among populations within groups, and within-population components for contrasting smooth bromegrass germplasm groups.

		Among groups		Among populations within groups		Within populations
Germplasm groups†	df	SS	*P*-Value	SS	*P*-Value	SS
		%		%		%
Among climatype and species	3	2.8	<0.0001	13.1	<0.0001	84.1
Smooth bromegrass vs. hybrids	1	3.6	0.0520	14.1	<0.0001	82.3
Meadow vs. steppe climatype	1	3.3	<0.0001	13.0	<0.0001	83.7
Meadow vs. intermediate climatype	1	0.1	0.4262	15.4	<0.0001	84.5
Steppe vs. intermediate climatype	1	1.8	0.0264	12.9	<0.0001	85.3
Land races vs. contemporary	1	0.1	0.3490	14.7	<0.0001	85.2
Selected vs. non selected for IVDMD	1	1.5	0.0020	14.1	<0.0001	84.4
Northern vs. southern origin	1	1.1	<0.0001	12.5	<0.0001	86.4
Eastern vs. western origin	1	0.0	0.4130	13.1	<0.0001	86.9

† Cultivars or populations belonging to each group are defined in Table 1.

of significant phenotypic changes from morphological studies reflects the underlying genetic similarity revealed by RAPD marker variation between contemporary germplasm and land races.

The intergroup phenotypic variation of meadow vs. steppe climatype accounted for 3.3% ($P < 0.01$) of the variation, reflecting some genetic differences between the two main smooth bromegrass climatypes (Table 6). In addition, the intermediate populations were closer to the meadow than steppe climatype, reflecting the northern origin (deriving from the meadow climatype) of the four intermediate populations in this study. Despite the low intergroup variation, there were differences among the smooth bromegrass germplasm groups at $P < 0.01$ as determined by AMOVA (Table 6). The genetic variation of RAPD markers among populations within land race cultivars and contemporary germplasm groups was the highest (14.7%), suggesting that contemporary germplasm sources as well as land race cultivars possess populations with high genetic potential for germplasm improvement and cultivar development. These results support previous studies (Casler et al., 2000, 2001), which demonstrate that smooth bromegrass original cultivars (land races) remain useful for germplasm improvement and cultivar development (Table 6).

The intergroup RAPD variation of the two latitudinal origins of smooth bromegrass germplasm (north and south) was 1.1%. The intergroup RAPD marker variation for smooth bromegrass populations developed east vs. west of 95°W longitude was not statistically significant. Most of the variability resides among populations developed, collected, or increased north vs. south of 42°N latitude in North America (Table 6). In terms of genetic variation as measured by analysis of molecular variance, latitude and latitude-related climatic and edaphic factors were more important than longitude and longitude-related factors. These results support previous studies that have shown latitude to be an important factor regulating genotype × location (GL) interactions for forage yield of smooth bromegrass (Knowles and White, 1949; Thomas et al., 1958; Casler et al., 2001).

The intergroup RAPD variation of populations selected vs. unselected for high IVDMD contributed significantly to the total variation ($P < 0.01$), reflecting genetic mean variation between the two germplasm groups (Table 6). These results suggest that differences in marker frequencies in those two groups of germplasm reflect some genetic basis for changes in phenotype of populations selected for IVDMD. Phenotypic changes in IVDMD represent one of the greatest gains in smooth bromegrass breeding program during the last 40 yr (Casler et al., 2000). Contemporary germplasm sources selected for IVDMD have resulted in genetic improvement in smooth bromegrass forage nutritive value, as demonstrated by their superiority in field trials over several locations (Casler et al., 2000, 2001). The experimental germplasm from Lincoln (Lincoln-HDMD-C3 and Lincoln-HDMDYD-C3) and the two crosses between high-IVDMD germplasm from the Nebraska and Wisconsin programs (WB19e and WB20e) have supported previous observations that IVDMD can be readily improved in smooth bromegrass by recurrent selection (Carpenter and Casler, 1990; Vogel et al., 1996; Casler et al., 2000).

Although the populations under study cover several interesting germplasm comparisons, and a wide range of the genetic variability available for breeding, the within-population variation was large due to the outcrossing reproduction and the octoploidy of smooth bromegrass. The level of partitioning of RAPD variation is dependent on the material under study and on breeding system of the species. Autogamous species in general have relatively low within-population variation (43% for *Hordeum spontaneum* K. Koch) (Dawson et al., 1993), while allogamous species have a higher percentage of within-population variation (73.8–94.9% for *Eucalyptus globulus* Labill.) (Nesbitt et al., 1995). The patterns of variation observed in this study were similar to those found in several studies of allogamous grass species, including smooth and meadow bromegrass (Ferdinandez et al., 2001), blue grama [*Bouteloua gracilis* (H.B.K.) Lag. ex. Steud.] (Phan, 2000), buffalograss (Peakall et al., 1995; Huff et al., 1993), and perennial ryegrass (*Lolium perenne* L.; Huff, 1997) where within-population variation was much higher than between-population variation. The within-population variation, which ranged from 72.9 to 80.5%, was consistent with geographic origins in buffalograss, an allogamous species (Huff et al., 1993). Phan (2000) found that the within-population variation accounted for 96.6% of the total variation in the plant collections of blue grama, while the within-population variation for bromegrass species ranged from 65.8 to 85.2% (Ferdinandez et al., 2001).

Genetic Relationships among Populations

A dendrogram based on distance calculated from 97 RAPD bands, did not segregate populations into distinct groups based on climatype or selection history (Fig. 1). There was, however, moderate correspondence to a previous morphological and anatomical clustering analysis on twenty-seven populations of smooth bromegrass (Casler et al., 2000). The Mantel test of matrix correlation between morphological/agronomic and molecular distances ($r = 0.55$) was significant at $P < 0.05$ with 95% confidence limits (0.52–0.24). Correspondence was particularly obvious for lines that are closely related to each other (e.g., WB19e and WB20e, Lincoln-HDMD-C3 and Lincoln-HDMDYD-C3) (Fig. 1 vs. Fig. 1 of Casler et al., 2000). WB19e and WB20e, two strains crosses between high-IVDMD germplasm from the Nebraska and Wisconsin programs were the two closest populations in the dendrogram.

Lincoln-HDMD-C3 and Lincoln-HDMDYD-C3, two experimental populations developed from land race 'Lincoln' after three cycles of selection for increased IVDMD, were genetically similar. Lincoln-HDMD-C3 and Lincoln-HDMDYD-C3 were very distant from Lincoln. These large genetic distances observed in the dendrogram between the two Lincoln selections and Lincoln suggests that many loci are involved in the difference between Lincoln and these selections. Differ-

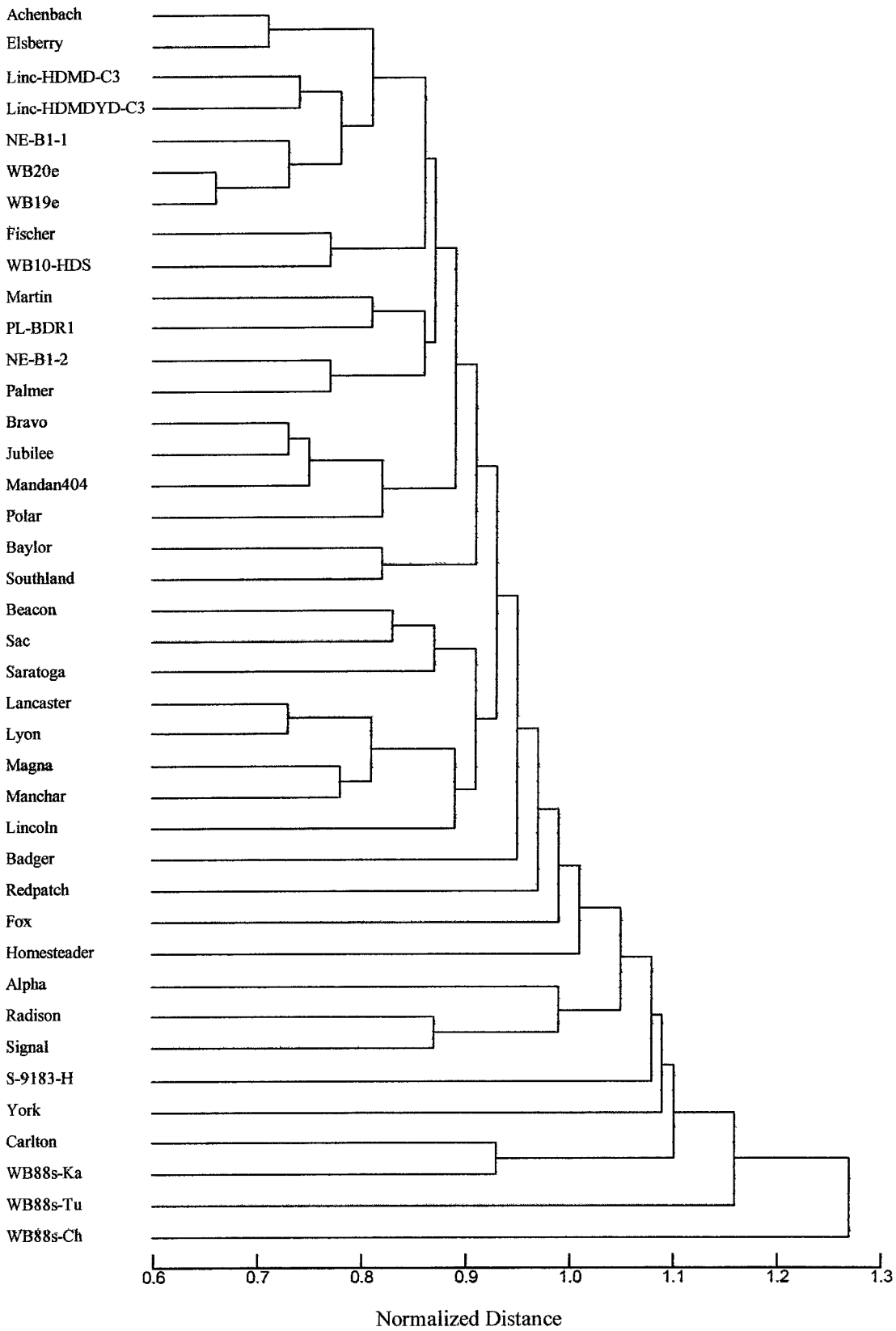

Fig. 1. Dendrogram constructed from 97 random amplified polymorphic DNA markers observed on 40 smooth bromegrass populations, based on Euclidean distances using the UPGMA clustering algorithm.

ences in IVDMD between Lincoln and these selections (Casler et al., 2000) are likely polygenic. Several studies in forage crops have shown a strong and consistent relationship between digestibility and lignin. The complex biosynthesis pathway in lignin composition and concentration involved many regulatory and structural genes (Boudet et al., 1995; Casler, 2001). These results support observations from previous studies that IVDMD is controlled by many genes with small effects and can be readily improved by recurrent selection (Carpenter and Casler, 1990; Casler et al., 2000; Casler, 2001; Argillier et al., 2000).

Both 'Alpha' and 'Badger' smooth bromegrass were derived from a broad-based smooth bromegrass germplasm pool that included a large number of plant introductions and cultivars with unknown identity (Casler and Drolsom, 1992, 1995). Badger and Alpha are sister lines that differ only by selection for compatibility with alfalfa. However, these two sister lines appeared to be genetically very distinct in the dendrogram, reflecting changes in alleles frequency associated with selection pressure applied to Alpha for persistence in mixture with alfalfa or a genetic bottleneck effect.

Cluster analysis was useful in identifying relationships among land race cultivars of smooth bromegrass with unknown or diverse pedigree and also between some experimental germplasm sources (Fig. 1). Many cultivars and populations appeared in the dendrogram as distinct populations with individual genetic identity, supporting previous research on morphological and agronomic traits (Casler et al., 2000, 2001). 'Homesteader', a composite of five strains from fields 50 yr old, was very distinct in the dendrogram. However, several clusters and subclusters of land-race cultivars and/or contemporary germplasm sources were observed. 'Achenbach', developed by some selection in late 1890s on the Achenbach brothers' farm, Washington County Kansas, and 'Elsberry', derived from old bromegrass field in northwestern Missouri, are examples of subclusters of old cultivars created by seed multiplication of ecotypes or land races (Thomas et al., 1958; Casler et al., 2000). 'Lancaster' and 'Lyon', developed from selection work at the Nebraska Agricultural Experiment Station, formed another subcluster of land races.

Clustering revealed two categories of smooth bromegrass meadow climatypes. The cultivars Mandan 404, Jubilee, Bravo, Palmer, and Martin formed two small groups with relatively small genetic distances and a slightly larger distance connecting the groups. Carlton, a meadow climatype selected for increased forage and seed yields after polycross progeny testing at Saskatoon (Lawrence et al., 1995), along with three accessions collected in Russia (USDA-ARS, 1990) were the most genetically distinct of all the populations. Of the three intermediate climatypes, 'Magna' and 'Manchar' had a relatively low genetic distance, but 'Signal' was most closely related to 'Radisson' a steppe climatype. The similarity between Signal and Radisson may reflect a similar selection history and selection environment. Both cultivars derive largely from Magna, but are genetically dissimilar to Magna (Fig. 1).

WB88S-Ch and WB88S-Tu, two natural ecotypes collected 80 km apart near Cherga and Tuekta in the Russian Altai Mountains (USDA-ARS, 1990), were the most genetically distinct of the 40 populations. These results were in concordance with the hypothesis that locales in which there is the largest amount of genetic variability are the centers of origin and domestication of crops (Vavilov, 1926, 1957; Mangelsdorf, 1953; Crow, 1992). Those centers of greatest diversity have great utility in the search for sources of new germplasm for plant improvement. Russia is believed to be an important center of origin of smooth bromegrass (Casler and Carlson, 1995; Vogel et al., 1996).

WB88S-Ka, another natural ecotype collected near Karavanniy, SE of Orenburg (SE of Moscow) (USDA-ARS, 1990), subclustered with the northern (meadow climatype) cultivar Carlton. Northern (meadow climatype) strains came from introductions from Russia during 1896-1898, while southern (steppe climatype) strains were shown to arise from French and Hungarian introductions around 1880 (Newell and Keim, 1943; Hansen, 1945), although, in the central Chernozem region in Russia, the steppe type was found with the meadow type (Vogel et al., 1996). Therefore, the close relationship between WB88S-Ka and Carlton may reflect a similar origin of these two germplasm sources. Carlton underwent relatively little selection for increased forage and seed yields after polycross progeny testing at Saskatoon (Lawrence et al., 1995). Moreover, Carlton had showed particular promise in a field study near Krasnoyarsk, also SE of Moscow, combining high green matter and seed yields (Kolchanova, 1989).

The cluster analysis did not reveal distinct separation between the interspecific hybrids and the rest of the smooth bromegrass cultivars and populations. Polar is a hybrid between *B. inermis* and *B. pumpellianus*, while S-9183-H is a hybrid between *B. inermis* and *B. riparius*. Because the majority of RAPD markers are thought to originate from the nuclear DNA (Weising et al., 1995), one might expect more distinct separation of the interspecific hybrids from the remainder of the smooth bromegrass cultivars and populations. There were no species-specific markers associated with any of the two inter-specific hybrids Polar and S-9183-H. The fact that the interspecific hybrids were not distinctively separated from the smooth bromegrass germplasm pool suggests that the contribution from *B. pumpellianus* and *B. riparius* genomes to the two hybrids is relatively small. One explanation could be due to the complex polyploid nature of *B. inermis. B. pumpellianus* and *B. riparius* may be progenitor candidates or very close to one of the *Bromus inermis* progenitors (Armstrong, 1991). *B. pumpellianus,* with excellent winter hardiness, is the most widespread of the five indigenous *Bromus spp*. in Alaska, and is closely related to *B. inermis*, with which it readily hybridizes (Klebesadel, 1984). Polar (*B. inermis* × *B. pumpellianus*) is a 16 clone synthetic; 11 clones were *B. inermis* × *B. pumpellianus* hybrids and five clones were *B. inermis*.

Another explanation could be that most of the *B. pumpellianus* and *B. riparius* chromosomes have been

eliminated as a result of mispairing during meiosis. *Bromus riparius* is relatively distant from *B. inermis* (Ferdinandez et al., 2001). Moreover, selection for the smooth bromegrass phenotype probably reduced the contribution of the *B. riparius* genome to the hybrid S-9183-H (Knowles and Baron, 1990; Ferdinandez et al., 2001). In addition, the number of individual plants assayed in this study for hybrid S-9183-H was the lowest due to poor germination. Therefore, sample size may be another reason why this hybrid did not exhibit any meadow bromegrass specific marker, as observed in other interspecific hybrid individuals between meadow bromegrass and smooth bromegrass (Ferdinandez et al., 2001). Also, Thormann et al. (1994) found RAPD data to be less reliable than RFLP data in estimating genetic relationships at the interspecific level.

Although the dendrogram did not indicate a clear pattern of division based on discrete or putative climatic or adaptation zones, as seen in some other crops (Gunter et al., 1996), the high genetic dissimilarity among the forty smooth bromegrass cultivars and experimental populations was clearly demonstrated by cluster analysis. There were few distinct clusters of smooth bromegrass cultivars and populations based on pedigree, climatype, or origins. Genetic distances derived from RAPD markers appear to be more reliable than pedigree or geographic origin information data for identifying germplasm with similar or different genetic backgrounds, i.e. genetic diversity cannot be equated to or assumed from differences in pedigree or origin. The large genetic distances between some populations of similar origin and pedigree suggest that genetic diversity has not been eroded by 50 yr of smooth bromegrass breeding in North America.

RAPD markers appear to be a valuable tool for assessing genetic diversity levels in smooth bromegrass. Genetic distances among cultivars, based on RAPD markers, were broadly concordant with genetic distances based on morphological and agronomic traits. Individual RAPD markers were highly discriminatory among populations, germplasm groups, and geographic origins, and demonstrated associations with an important agronomic trait (IVDMD). There is a considerable level of genetic variability within smooth bromegrass populations, both land races and contemporary populations, that has yet to be utilized in breeding new cultivars.

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